An infrared diagnostic system to detect causal agents of grapevine trunk diseases

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ABSTRACT

In most vineyards worldwide, agents of grapevine trunk diseases represent a real threat for viticulture and are responsible for significant economic loss to the wine industry. The conventional microbiological isolation technique used to diagnose this disease is tedious and frequently leads to false negatives. Thus, a dire need exists for an alternative method to detect this disease. One possible way involves infrared spectroscopy, which is a rapid, nondestructive analytical tool that is commonly used for quality control of feed stuffs. In the present work, a midinfrared spectrometer was tested as a fast tool for detecting agents of grapevine trunk disease. Midinfrared spectra were collected from 70 *Vitis vinifera* L. cv. Cabernet Sauvignon one year old trunk wood samples that were infected naturally in one viticulture nursery of the south of France. The samples underwent polymerase chain reaction and morphological identification, and the results were correlated to the midinfrared spectra by using multivariate analysis to discriminate between noninfected and infected samples. Based on comparison with some control samples, the highest percentage of correct identification of fungal contamination when using the midinfrared spectroscopy method is 80%.

1. Introduction

The progression of grapevine trunk diseases (GTDs) that is currently occurring in most vineyards worldwide represents a major threat to viticulture and is responsible for significant economic loss to the wine industry. The decrease in productivity is due to externalized symptoms and the resulting early decline of plants in the field leads to a loss of wine typicality (Laveau et al., 2009).

The GTD Esca is caused by xylem inhabiting *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* in association with several other fungi (Bertsch et al., 2013; Mugnai et al., 1999). In addition, a number of *Botrysphaeria* species have been identified as causal agents of Black Dead Arm (BDA) (Larignon et al., 2009). The more prevalent fungal species in France are *B. obtusa* and *B. parva*. Another fungus, *Eutypa lata*, causes Eutypa dieback (Rolshausen et al., 2006). Pathogens are thought to be present on the surface of canes and to infect plant material during soaking and stratification at the nursery and during vineyard management (Pouzoulet et al., 2013a). They attack perennial organs, leading to extensive inner necrosis in the trunk and arms (Mugnai et al., 1999; Rolshausen et al., 2006; Urbez Torres et al., 2006). The symptoms of the disease appear several years after the first infection. GTD fungi have in common a slow growth and induce an extremely complex and variable symptom expression, making GTD difficult to diagnose in vineyards. Current methods to classify fungal species rely on morphological characteristics such as, in particular, reproductive structures. These methods are fastidious and frequently lead to false negatives because the pathogens are often overgrown by other microorganisms in semi selective media (Aroca and Raposo, 2007).

Numerous studies have focused on molecular based identification within a single fungal family (Aroca and Raposo, 2007) or detection of GTD fungi (Pouzoulet et al., 2013a). The cost and time required for such analytical methods limit their use for rapid screening of raw materials. Thus, a dire need exists for an alternative method, such as infrared spectroscopy, which would allow a fast screening of grapevine wood. This method would be similar to the use of infrared spectroscopy to analyze the quality of food and feed, which is now routine (Bertrand and Dufour, 2005). The use of infrared spectroscopy would also benefit from the studies that have identified the various fungi and yeasts responsible for different fungal diseases (Costa et al., 2007; Erukhimovitch et al., 2005; Fischer et al., 2006; Graeff et al., 2006; Huang et al., 2007; Levasseur Garcia, 2012; Mariey et al., 2001; Peiris et al., 2010; Sankaran et al., 2012). For more information on the various infrared spectroscopic techniques for detecting plant diseases, please see the review by Sankaran et al. (Sankaran et al., 2010). To date, however, no study has used infrared spectroscopy to discriminate between GTD infected and noninfected grapevine trunks. Therefore, to address

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this need, the present study examines 70 naturally infected wood samples to investigate the potential of midinfrared (MIR) spectroscopy to detect GTD.

2. Materials and methods

2.1. Plant material

70 samples from one year old canes of *Vitis vinifera* L. cv. Cabernet Sauvignon clone 15 were used. The samples were collected from only one nursery, located in the Gaillac vineyard, (Midi Pyrénées) in the south of France. The samples are grafted plants, grown open air. After harvest, they were stored in the dark at 4 °C.

2.2. DNA extraction

The surface of the sample was cleaned by removing bark around the graft union and at the basal end of the rootstock, using a sterile scalpel in sterile conditions. Each sample is constituted by 5 wood sections of the same grafted plant, collected all along the plant to be representative of the whole plant. Each wood section size measures few millimeters of diameter and one millimeter on thickness. Wood sections were cut with clippers disinfected with 70% ethanol. Samples were lyophilized over 24 h and grinded at room temperature. Powder was incubated at 65 °C in a modified cetyltrimethyl ammonium bromide (CTAB) extraction buffer from Doyle and Doyle (Doyle and Doyle, 1987). 24:1 chloroform:isoamyl alcohol solution was added and the mixture was incubated on ice. Total DNA extraction was done by using a DNeasy plant mini Kit (Qiagen, USA) and following the manufacturer protocol. The final elution volume was 50 μl, and the samples were stored at −20 °C.

2.3. Primer design and sequence alignment

Primer sets were checked using Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/primer3/). Multiple sequence and primer alignments were performed with Multalin software (http://multalin.toulouse.inra.fr/multalin.html).

2.4. Qualitative PCR assays

PCR reactions were carried out in 25 μl reaction mixture containing: 12,5 μl of 2× Plexor™ Master Mix (Promega, Cat. No. A4031, Madison, USA), 0,2 μM of each primer, 10 ng of purified genomic DNA. Reaction conditions were: initial denaturation (94 °C 5 min) followed by 35 cycles of denaturation (94 °C, 30 s), primer specific annealing temperature for 1 min and elongation (72 °C, 1 min), and a final elongation for 5 min at 72 °C. The amplified products were examined by polyacrylamide gel 4 12% (Invitrogen, Carlsbad) electrophoresis.

2.5. Quantitative real time PCR (rt PCR) assays

Reactions were carried out in a final volume of 25 μl reaction mixture containing: 12,5 μl of 2× Plexor™ Master Mix (Promega, Cat. No. A4031, Madison, USA), 0,3 μM of each primer. Labelled Plexor primers (5′ MeisodC) were synthesized by Eurogentec S. A (5′FAM labelled, ref.: PB PP001 004; 5′TAMRA labelled, ref.: PB PP100 004; 5′ROX labelled, ref.: PB PPOO16 004; Liege science park, Seraing, Belgium) and were diluted in MOPS/EDTA buffer (Promega, Cat. No. Y510A, Madison, USA). Unlabelled primers were synthesized by Invitrogen (ref:10336 022, Fisher Bioblock Scientific, Illkirch, France) and diluted in nuclease/nucleic acid free water (Promega, Cat. No. P119A). A maximum of 2 μl of DNA template per reaction. Experiments were conducted with an ABI 7500 real time PCR (rt PCR) cycler using the software ABI SDS version1.4 with the default settings. The cycling program consisted of (i) an initial denaturation step at 95 °C for 5 min, (ii) forty 5 s cycles at 95 °C for denaturation, followed by 35 s at 65 °C for both annealing and extension, and (iii) an additional melting analysis of 40 min from 60 to 95 °C. The data were analyzed by using Plexor® Analysis software version 1.5.6.2 (Promega), and melt curve thresholds were fixed to 20% of their respective 10^4 standards.

2.6. Conventional microbiological isolation

Five wood sections of the same grafted plants were collected all along the plant, on the neighborhood of the 5 wood previous sections for DNA extraction, to be representative of the whole plant. Each wood section size measures few millimeters of diameter and one millimeter on thickness. Wood sections were cut with clippers disinfected with one spray of 70% ethanol and plated at 26 °C in the dark in Petri dishes containing potato dextrose agar (Merck, Germany, Cat. No. 1 10130 0500). Fungi were detected and enumerated after 10 days. The fungal isolates were identified as Pch, Bob, Pal, or Bpv (referring to *Phaeomoniella chlamydospora, Botrysphaeria obtusa, Phaeoacremonium aleophilum* and *Botrysphaeria parva* respectively) on the basis of their morphological characteristics.

2.7. Fourier transform infrared spectroscopy measurements

Fourier transform infrared (FTIR) spectra of wood powder were measured by means of an Avatar FTIR spectrometer (Thermo Electron Corporation, USA). The spectral range spanned from 400 to 4000 cm^{-1} with a resolution of 2 cm^{-1} after 32 scans. Finely divided 1 mg samples of wood were ground and dispersed in a matrix of 150 mg KBr, followed by compression to form pellets.

2.8. Preprocessing of spectra

Spectra were preprocessed to remove the effects of light scattering and to compensate for baseline offset and bias. To obtain the best discrimination model, seven different types of spectral preprocessing were tested. These included no treatment (raw data), the first and second derivative Savitzky Golay methods (D1 and D2, respectively), standard normal variate (SNV) ± detrending, and SNV ± D1 or D2. Derivatives were used to emphasize small bands and to resolve overlapping peaks. SNV removes the multiplicative interference due to scatter and particle size and corrects the variation in baseline shift (Sun, 2009). Spectral preprocessing was done by using the Unscrambler® Multivariate Data Analysis software (version X; CAMO A/S, Oslo, Norway).

2.9. Multivariate data analysis

Clustering analysis was used to determine the number of clusters that better define the wood dataset based on fungal contamination (percent contamination by Pch, Bob, Pal and Bpv). The K means algorithm was used because of its simplicity (Tuffery, 2007): the only parameter that must be defined is the number of clusters to be located. Statistical analyses were done with XLSTAT version 2014.5.02 (Addinsoft, New York, USA).

After preprocessing, a principle components analysis (PCA) was done to compress the spectra into a set of linearly uncorrelated variables called principal components (PCs). The first PC accounts for the maximum variability in the MIR spectra, with subsequent PCs accounting for ever decreasing variability (Tuffery, 2007). PCA was done with the Unscrambler® Multivariate Data Analysis software (version X; CAMO A/S, Oslo, Norway).

A chi squared automatic interaction detector (CHAID) decision tree was used to build a discrimination model. The quality of the model was evaluated by calculating prediction accuracy and classification errors from confusion matrices (Visa et al., 2011). A confusion matrix was used. The *true positive* rate (TP) is the proportion of positive cases that were correctly identified; the *false positive* rate (FP) is the proportion

Table 1
Fungal contamination of 70 wood samples (rt-PCR).

rt-PCR	Mycoflora content of 70 wood samples (number of copies)			
	Minimum	Maximum	Mean	Occurrence (%)
Pch	0	4967	696	47
Bob	0	1967	53	31
Pal	0	6267	225	13
Bpv	0	1800	28	4
Total	0	6947	1002	64

Pch, Bob, Pal and Bpv refer to *Phaeomoniella chlamydospora, Botrysphaeria obtusa, Phaeoacremonium aleophilum* and *Botrysphaeria parva* respectively.

of negatives cases that were incorrectly classified as positive; the *true negative* rate (TN) is defined as the proportion of negatives cases that were classified correctly and the *false negative* rate (FN) is the proportion of positives cases that were incorrectly classified as negative (Fawcett, 2006).

The prediction accuracy and the classification error can be calculated from this matrix. The prediction accuracy is obtained as follows (Eq. (1)):

$$\text{Accuracy} \quad (TP + TN)/(\text{total}) \tag{1}$$

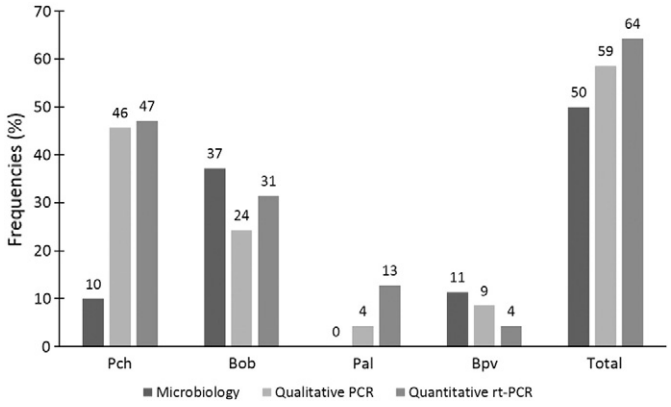

Fig. 1. Occurrence of fungal contamination of 70 wood samples.

The classification error is obtained from the confusion matrix (Eq. (2)):

$$\text{Error} \quad (FP + FN)/(\text{total}) \tag{2}$$

The best model is the one with the highest accuracy and the lowest error.

The results were analyzed by using a one way analysis of variance (ANOVA) at $p \leq 0.05$, and Tukey's test (McGill et al., 1978) with XLSTAT version 2014.5.02 (Addinsoft, New York, USA).

3. Results and discussion

3.1. Fungal contamination of grapevine trunk

Seventy wood samples were characterized in terms of their fungal contamination. The fungal count was simultaneously determined by qualitative PCR and quantitative rt PCR and by identifying fungal species in microbiology. The results are presented Table 1 and Fig. 1.

Table 1 summarizes the fungal contents. The mean total fungal content of the samples was 1002 copies, with extreme values ranging from 0 to 6947 copies, which are typical values for this type of sample.

The wood samples were contaminated by fungi at the rate of 64%, 59%, and 50% as determined by rt PCR, PCR, and microbiological methods, respectively. The wood samples were mainly infected by Pch, which appeared in 47%, 46% and 10% of the samples, as per rt PCR, PCR and microbiological, respectively. The most prevalent species were Pch and Bob. Pal, however, was not detected by the microbiological methods. Under these experimental conditions, detecting Pal was difficult because it developed more slowly than the other fungi, which used this advantage to directly compete with Pal. Thus, observing Pal was rendered more difficult. Microbiological methods thus detected less contamination for Pch and Pal. The opposite situation occurred for Bob and Bpv.

Grapevine wood had several contamination profiles. A classification was used to specify these groups. The model consisted of a nonsupervised k means classification with determinant *w*, which allowed the wood to be grouped into six distinct profiles. Fig. 2 shows the average contamination rates of each of the six groups.

Profile 1 included samples of noncontaminated wood and represented 36% of the samples (25 out of 70 samples). Pal, Bob, and Pch were the major contaminants in profiles 2 (11%), 3 (11%), and 6 (36%),

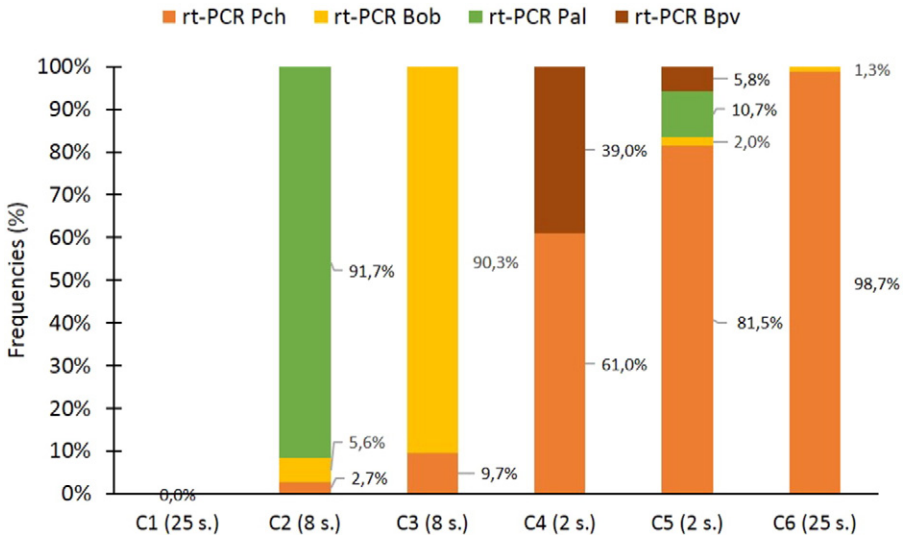

Fig. 2. Fungal contamination profiles of 70 samples (s.) of grapevine trunks. (Class 1: non-contaminated samples - Class 2: Pal major contaminant - Class 3: Bob major contaminant - Class 4: Pch and Bpv major contaminants - Class 5: Pch and Pal major contaminants - Class 6: Pch major contaminant) (Pch, Bob, Pal and Bpv refer to *Phaeomoniella chlamydospora, Botrysphaeria obtusa, Phaeoacremonium aleophilum* and *Botrysphaeria parva* respectively).

respectively. Profiles 4 and 5 contained wood samples contaminated by two fungal species: Pch and Bpv for profile 4 and Pch and Pal for profile 5. However, these two profiles accounted for only 6% of the samples (4 out of 70).

3.2. Sample spectra

Fig. 3(a) shows the raw MIR spectra for the six different classes and Fig. 3(b) shows the average first derivative MIR spectra of infected and noninfected wood.

Fig. 3(a) shows that the infrared absorption peaks overlap each other. In Fig. 3(b), the main features appear between 3400 and 3100 cm^{-1} and between 1800 and 800 cm^{-1}. Previous studies determined the spectral zones that are modified by the presence of fungi (Kummerle et al., 1998; Lee and Chapman, 1986; Naumann et al., 1988; Naumann et al., 1991). These spectral signatures were mainly the result of the total cell chemical composition of fungal strains and wood; for instance, the lipids, nucleic acids, proteins, polysaccharides and especially the modified protein or carbohydrate levels of wood. Fackler et al. studied the degradation of lignin in infected wood (Fackler et al., 2007). In this work, the main spectral modifications occurred at 1596, 1505 and 1462 cm^{-1} and were all assigned to lignin; features at 1738, 1158, 1375 and 898 cm^{-1} were all assigned to hemicellulose and cellulose. Faix et al. (1991) reported a spectral modification at 1646 cm^{-1}, which was assigned to lignin (Faix et al., 1991). These spectral modifications corresponded to the main modifications found in our MIR spectra in the range 1800 800 cm^{-1}, which were assigned to the degradation of the major wood constituents. Features

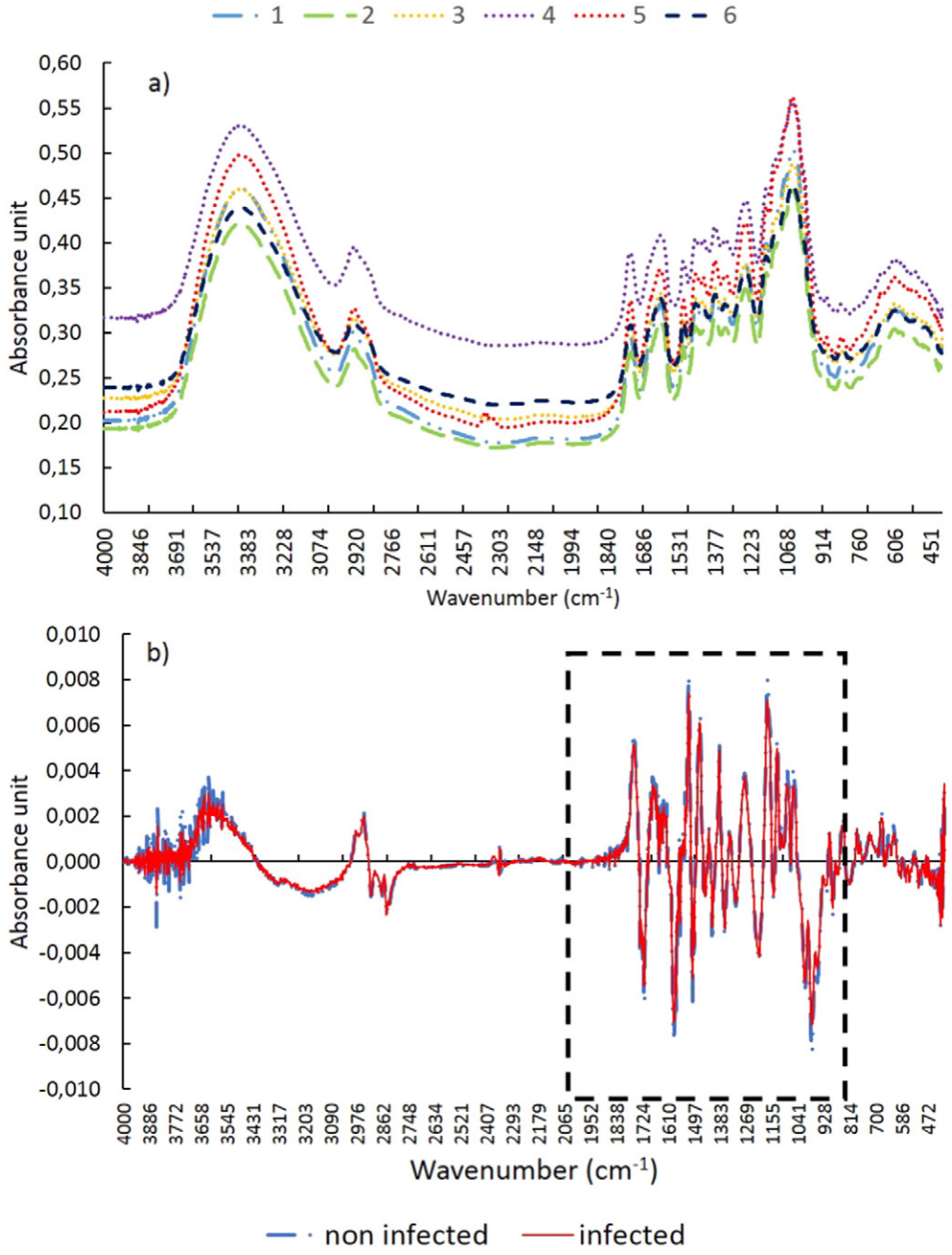

Fig. 3. Average raw (a) and first-derivative (b) MIR spectra of infected and noninfected wood. (Class 1: non-contaminated samples - Class 2: Pal major contaminant - Class 3: Bob major contaminant - Class 4: Pch and Bpv major contaminants - Class 5: Pch and Pal major contaminants - Class 6: Pch major contaminant) (Pch, Bob, Pal and Bpv refer to *Phaeomoniella chlamydospora, Botrysphaeria obtusa, Phaeoacremonium aleophilum* and *Botrysphaeria parva* respectively).

Table 2
CHAID classification (percent correct) for fungal contamination of grapevine trunk.

Data pretreatment	Calibration (n = 65)			External validation (n = 5)		
	Microbiology	Qualitative PCR	rt-PCR	Microbiology	Qualitative PCR	rt-PCR
SNV + D2	56	67	70	40	40	**80**
SNV + D1	63	**76**	72	20	60	**80**
SNV[c]	66	69	75	40	40	**80**
D2[b]	69	75	73	**60**	**80**	60
SNVD[d]	69	73	73	**60**	60	**80**
D1[a]	70	72	73	**60**	60	**80**
Raw	**72**	**76**	**76**	**60**	60	**80**

Bold type indicates the best classifications obtained for each method (microbiology, qualitative PCR, or rt-PCR).
[a] First derivative.
[b] Second derivative.
[c] Standard normal variate.
[d] Standard normal variate and detrending.

around 3400 cm^{-1} were assigned to infection by fungi (Gordon et al., 1998; Greene et al., 1992). Agrelli et al. (Agrelli et al., 2009) studied wood affected by Esca disease and noticed the destruction of hemicellu loses and noncrystalline cellulose along with modifications of the pectic (carbohydrates associated with polyphenols) and ligninic wood fractions.

3.3. Classification of wood samples as noninfected or infected

We used the CHAID algorithm to create a classification model to sep arate grapevine wood into two categories: noninfected by any fungi (Pch, Bob, Pal, or Bpv) or infected by at least one of these fungi. The seven first PCs obtained by PCA after each of the seven preprocessing steps were used as explanatory variables. We used three types of depen dent variables: noninfected infected as per microbiological methods, noninfected infected as per qualitative PCR, and noninfected infected as per rt PCR. Only the total level of contamination was studied. Forty two different models were then developed. For each model, 65 samples were used to develop the model and the same five samples, randomly chosen, were used as control samples. The results of applying this model are presented in Table 2.

As shown in Table 2, the highest percentage of correct identification of fungal contamination in external validation was 60%, 80%, and 80%, for microbiological methods, qualitative PCR, and rt PCR, respectively.

The raw spectra and preprocessing by SNVD and D2 produced the most accurate models ($F = 4.095$, p value $= 0.018 < 0.05$). Similarly, using the PCR (both qualitative and rt PCR) results produced the highest percent of correct identification of fungal contamination ($F = 12.933$, p value $= 0.001 < 0.05$). Detection by microbiological methods was made difficult by the prevalence of certain species and the concom itant errors in interpretation that this could engender. In contrast, PCR (both qualitative and rt PCR) allowed molecular diagnostics that leave little room for errors in interpretation (Pouzoulet et al., 2013b). Qualita tive PCR can detect the presence of fungi in samples, and rt PCR can quantify the number of copies in the initial sample with a precision of up to three copies.

Because the wood was infected naturally in our study, we obtained a reliable picture of what occurs in the field. For example, the concentra tion ranges were representative of what was found in the field. Our best models offered an accuracy of 60% (microbiology) to 80% (PCR). This study thus fills a gap in the literature, which, to our knowledge, contains no studies that use infrared spectroscopy and focus on these fungi. The largest magnitude spectral change caused by fungal contamination oc curred in the ranges 4000 3657, 3500 3300, 2800 1900, and 950 400 cm^{-1}, as observed by several other groups (Agrelli et al., 2009; Fackler et al., 2007; Faix et al., 1991; Gordon et al., 1998; Greene et al., 1992; Kummerle et al., 1998; Lee and Chapman, 1986; Naumann et al., 1988; Naumann et al., 1991). The interpretation of these observations was based on the hypothesis that detecting fungi is linked to a complex ensemble of information related to growth of the fungus on wood. The spectra sum up the macromolecule content of fungi (proteins, nucleic acids, lipids, polysaccharides, etc.) to produce a fingerprint that can be used for differentiating fungi (Lee and Chapman, 1986) through cluster ing. The combination of infrared spectroscopy and multivariate tech niques could be used in standard laboratory applications and could serve to support diagnostic efforts.

4. Conclusion

This study assessed the feasibility of using infrared spectroscopy to screen grapevine wood for fungal contamination. The spectral signa tures we observed were related to the presence of fungi on the wood. We obtained up to 80% correct identification of infected plants, which can be very useful, especially considering the rapidity and simplicity of the method. For example, this qualitative approach could help nurseries sort their vine plants and remove infected plants. The advantages of this approach are minimum sample preparation, rapid analysis (<1 min) compared to DNA analysis, a reagent free procedure, reliability of the diagnosis, and relatively low cost. In addition, it requires only a small quantity of wood (1 mg) and has minimal technical requirements. These models were promising. The next step is to add further fungi iso lates to the database, fungi from different cultivars, environments where the vines are grown, and vine age. Indeed, the power of the method depends directly on the quality of the library of reference spectra.

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